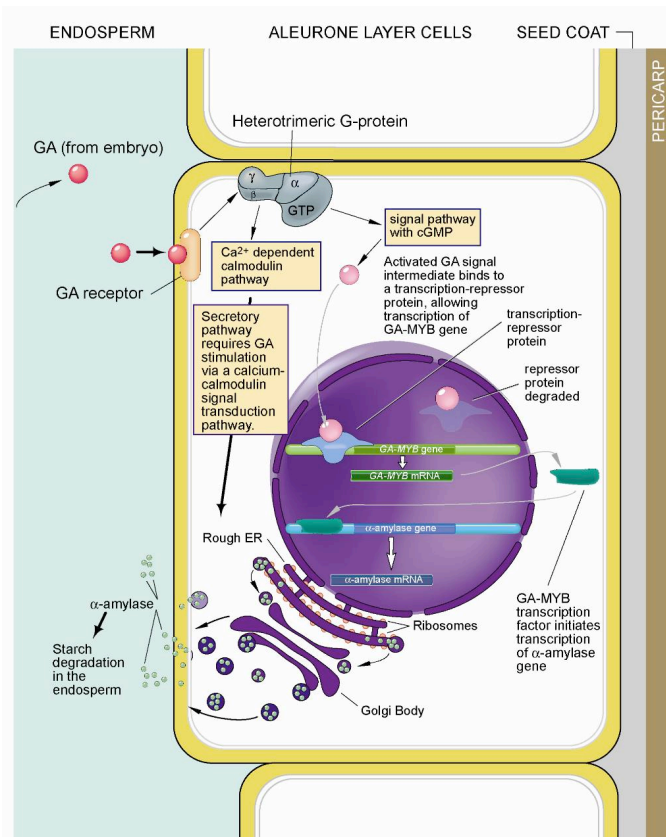
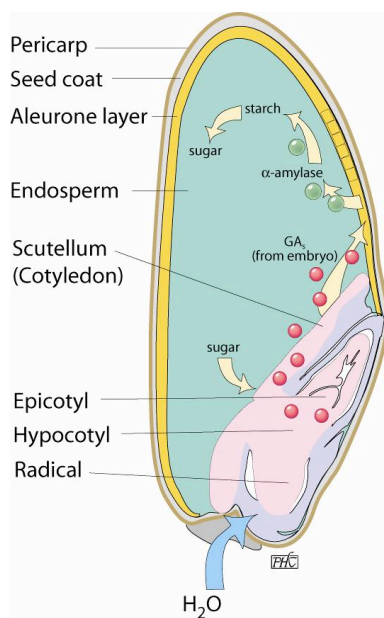


INDUCTION OF ALPHA-AMYLASE SYNTHESIS BY GIBBERELLINS

Objectives:

Introduction:

Synthesis of hydrolytic enzymes in the barley aleurone layer is controlled by gibberellins that are secreted by the embryo at the time of germination. Alpha-amylase has been investigated particularly well, and it has been shown that hormone treatment of isolated aleurone layers induces *de novo* synthesis of the enzyme. The effect of gibberellins on embryo-less half seeds can be demonstrated either by assaying for α -amylase or by measuring the level of reducing sugars liberated in response to hormone treatment. Either test can be used as a bioassay for gibberellins.



We will investigate the induction of alpha-amylase expression in germinating barley seeds. We will study the kinetics of alpha-amylase induction by analyzing this enzyme at different times during seed germination (Experiment 1). We will also study the induction of alpha-amylase by gibberellins seeds embryoless seeds (Experiment 2).

Question 16: Why do we study the effect of GA on alpha-amylase in embryoless seeds and not on whole seeds?

Protocols:

Students will work in pairs

PART 1: Alpha-amylase induction

TA for Thursday:

Make sure to turn on the sterile hoods at the beginning of class.

On Thursday TA will provide each group with:

- * ~160 (40 +120) barley seeds
- * 2 x 50 ml tube (falcon tube), 1 for experiment 1 and 1 for experiment 2
- * ~100 ml 20% bleach
- * 500 ml sterile water
- * 6 sterile glass petridish with 1 whatman #3 or 2 pieces whatman #1 or #2
- * 1 Sterile 10 ml pipette
- * 1 orange pipettor

EXPERIMENT 1: Alpha-Amylase activation during germination.

1. Seed preparation (Thursday):

~40 barley (cv Himalaya) seeds are sterilized with 20% commercial bleach for 5 minutes. Thereafter the seeds are washed 4-5 times with sterile water. The sterilized seeds (10 seeds per plate) are imbibed in three 10 cm Petri dishes containing filter paper and 7 ml sterile water. The petridishes will be placed by the TA at 4C. The TA will take out 1 plate on Saturday, 1 on Monday and you will take one out on Tuesday and place them in a drawer. Make sure you label the plates with your name and date and experiment number.

EXPERIMENT 2: Alpha-amylase induction by GA

1. Seed preparation (Thursday):

Sixty barley (cv Himalaya) seeds are cut in half perpendicular to the longitudinal axis, and the embryo portion is discarded. The embryo-less halves are sterilized with 20% commercial bleach for 5 minutes. Thereafter the half seeds are washed 4-5 times with sterile water. The sterilized half-seeds are imbibed in three sterile 10 cm Petri dishes containing filter paper and 7 ml sterile water. The Petri dishes are incubated at room temperature for 60-70 h. Due to timing restrictions the TA will place your plates in the cold and take them out on Saturday and put them in a drawer. Make sure you label your plates with your name, date and experiment number.

2. Treatments (Tuesday):

Material:

TA: give each group

- * 20 ml of incubation buffer; 1mM Acetate buffer (pH 4.8), 20 mM CaCl₂, to make 1L:
weigh 0.041 g Sodium acetate and 2.94 g CaCl₂, dissolve in 800 ml H₂O, add 30 ml acetic acid, check pH and adjust volume to 1 L with H₂O. Sterilize.
- * 1 aliquot of 300 ul of each:
 - GA₃ stock: 10 mM in 90% ethanol
 - Chloramphenicol 10mg/ml in 100% ethanol
 - 2 mg/ml cycloheximide

- * 90% ethanol for GA₃ dilutions
- * access to 10 µl pipette (there is Eppendorf one in the 255 cabinet)
- * access to 10 µl tips (get from Biochemstore or from Farre lab, 1 box sufficient for all groups)
- * 6x 15 ml plastic tubes
- * 2x Forceps
- * Flaming ethanol to sterilize the forceps
- * 1 petri-dish with endosperm halves and 1 petri-dish with embryo halves.

To prepare each of the following treatments, add the necessary amount of GA₃, cycloheximide and chloramphenicol to 1.5 ml incubation buffer (1mM Acetate buffer (pH 4.8), 20 mM CaCl₂, [provided by the TA](#)) in a 15 ml tube and mix well. 15 ml tubes are sterile, do not open them outside the hood. Prepare all the treatments aseptically in the sterile hood.

Treatments:

- | | |
|--|----------------------------|
| 1. Buffer control + ethanol* | + 10 µg/ml chloramphenicol |
| 2. Buffer + 50 nM GA ₃ | + 10 µg/ml chloramphenicol |
| 3. Buffer + 100 µM GA ₃ | + 10 µg/ml chloramphenicol |
| 4. Buffer +100 µM GA ₃ + 2 µg/ml cycloheximide | +10 µg/ml chloramphenicol |

Question 17: What are the volumes of GA₃, cycloheximide and chloramphenicol stocks you need to add to the incubation buffer to achieve these final concentrations?

Question 18: Why do we do treatment 4? What is the difference to treatment 3?

***Note:** buffer controls need to include the same volume of 90% ethanol used in treatment #4.

Cycloheximide is an inhibitor of eukaryotic protein synthesis (TOXIC !)

Note for students: to make the 50 nM GA₃ treatment, you will need to dilute your 10 mM GA₃ stock further (remember you cannot pipette accurately a volume smaller than 1.5 µl), prepare the new dilution using 90% ethanol.

Transfer 10 embryoless halves aseptically to each 15 ml vial. Incubate in a drawer for 2 days

PART 2: Preparation of protein extracts (Thursday)

Material, TA provide students with:

- * 2 ceramic mortar and pestel per group
- * 2 spatulas per group
- * 10x 2 ml microfuge tubes
- * 10x 1.5 ml microfuge tubes
- * 10 ml incubation buffer (1mM Acetate buffer (pH 4.8), 20 mM CaCl₂).

1.

- *For Experiment 1 seeds:* Transfer 5 seeds and 1.5 ml incubation buffer (1mM Acetate buffer (pH 4.8), 20 mM CaCl₂, see above) to a mortar and grind tissue for 1-2 minutes. Transfer extract to a 2 ml microfuge tube using a spatula and/or a pipette.
- *For Experiment 2 seeds:* Transfer the seeds and their respective incubation solutions to a mortar and grind for 1-2 minutes. Transfer extract to a microfuge tube using a spatula.

2. Centrifuge microfuge tubes with extract for 2 min at max. speed to remove debris.

Question 20: What is the “debris”?

3. Transfer supernatant to a new 1.5 ml tube (don't disturb the pellet). Save the supernatant for protein and enzyme assay.

4. Assay protein using the Bradford method.

Bradford assay:

Also known as the Bio-Rad or “dye-binding” assay. This assay has largely replaced the “Lowry” assay because it is faster, simpler and free of interference from commonly used biochemical reagents. One disadvantage is that the dye sticks to everything and glassware must be rinsed with ethanol after use. Other disadvantages are that many detergents interfere with color development and acidic proteins bind poorly.

The choice of standard protein is somewhat arbitrary- it should be inexpensive relatively pure, and not have an atypical response to the dye. Bovine serum albumin (BSA) or Bovine Gamma Globulin (IgG) are commonly used.

Reference: Bradford, M.M. Anal. Biochem. 72: 248
Bio-Rad Technical Bulletin 1069

Material to be provided by TA to each group:

- * 4 ml Fluka coomassie reagent #27813
- * sterile double distilled water
- * 500 µl 1mg/ml IgG standard (prepare from Farre's lab stock, in box at -20C)

1. Dilute the staining solution (Fluka coomassie reagent #27813) 1:10, make enough to measure all the standards and your extracts (see below).
2. Make a standard curve of 0, 0.1, 0.25, 0.5, 0.75 and 1mg/ml IgG. Prepare 100 µl of each concentration using 1.5 ml tubes.
3. Add 30 µl of each standard to 1 ml staining solution and mix well. Transfer this solution to 1 ml plastic cuvettes (1cm path length).
4. To analyze your samples dilute your extract 1:10, you will just need ~100µl of your diluted extract. Add 30 µl of each diluted extract to 1 ml of diluted staining solution (use 1.5 ml tubes) and mix well. Transfer this solution to 1 ml plastic cuvettes (1cm path length).
5. Measure the absorbance at 595 nm. Use your standard of 0 mg/ml IgG to blank your photometer.
6. Using Excel make a standard curve by plotting the concentration of your different standards on the Y-axis and their absorbances on the X-axis. Use a scatter plot and draw a trend line across your measurements. You will use the equation of this line to calculate

the protein concentration of your samples. How good is your standard curve? Print out your curve and add it to your notebook.

Note: If the absorbance of any of your samples is smaller than any of the values for your standards adjust the dilution of your extract accordingly.

PART 3: AMYLASE ENZYME ASSAY

We will measure the amount of alpha-amylase activity in your extracts by measuring how fast your extracts can degrade starch. The protein extracts will be incubated with a starch solution and its degradation measured using iodine staining of starch. Sigma A2771 Barley alpha-amylase will be used as a positive control at a concentration of 1 unit alpha-amylase per ml. One unit liberates 1 mg of maltose from starch in 3 min.

Solutions to prepare by TA:

1. 10 mM potassium phosphate buffer, pH 7 (see cloning manual book on how mix KH_2PO_4 and K_2HPO_4 to achieve the pH), autoclave.
2. Barley amylase standard. This amylase has 1.1 units enzyme (U) /mg solid and should be dissolved at 0.9 mg/ml in 10 mM potassium phosphate buffer pH 7.0; i.e. this equals a concentration of ~1U/ml. Note: pure alpha-amylase has a specific activity of approximately 1000U/mg protein and therefore the Sigma A2771 alpha-amylase is not highly purified. Give 3 ml for the 1U/ml standard to each group.
3. Iodine reagent (Jones & Varner, 1967): 1ml of I_2 -KI + 100 ml 0.05 N HCl, mix fresh before use. Give 30 ml to each group.

For 50 ml I_2 -KI stock:

Dissolve 3g potassium iodide in 50 ml of water, add 300 mg I_2 , stir until dissolved.

Be careful with iodine fumes, weigh under the hood !

4. Starch substrate:

for 500 ml :

0.75 g starch

3 g KH_2PO_4

200 μM CaCl_2

Add H_2O to 500 ml, mix well

Boil 1 minute

Cool, centrifuge 10 min at 3000g, decant and use clear supernatant.

Give 30 ml to each group.

5. Distilled H_2O : Give 100 ml to each group.

6. Timer (1 per group)

Assay:

1. Make different dilutions of enzyme (standards and extracts) in a total volume of 1 ml, using the 10 mM potassium phosphate buffer pH 7.0, then transfer to 13 x 100 mm test tube:

*For standards: make dilutions of 0 (this will be your water standard to be used to blank the photometer), 0.1, 0.25, 0.5, 0.75 and 1 U/ml.

* For extracts: use 30 µl extract in 1 ml.

2. Start the reaction by adding 1 ml of starch substrate
3. Stop reaction after 2 minutes by adding 1 ml of iodine reagent
4. Add 3 ml of distilled H₂O, mix and read absorbance at 620 nm.
5. Using Excel make a standard curve by plotting the U/ml of your different standards on the Y-axis and their absorbances on the X-axis. Use a scatter plot and draw a trend line. You will use the equation of this line to calculate the alpha-amylase activity of your samples. How good is your standard curve? Print out your curve and add it to your notebook.

Question 21: Why is the decrease in absorbance proportional to the quantity of alpha-amylase in the reaction mixtures?

Note: If your absorbance for any of your extract preparations is lower or higher than the range of values from your standard curve, use more or less extract accordingly for your sample preparation and repeat the assay. **Question 22: Why?**

Note: The color should be still light blue after stopping the assay. If it remains dark blue, not enough enzyme was used or the time of incubation was too short.

PART 3: WESTERN-BLOT

(from Sambrook and Russell (2001). Molecular Cloning, A Laboratory Manual. CSHL Press, Cold Spring Harbor, NY. 3rd Edition):

"Western blotting [...] is to proteins what Southern blotting is to DNA. In both techniques, electrophoretically separated components are transferred from a gel to a solid support and probed with reagents that are specific for particular sequences of amino acids (western blotting) or nucleotides (Southern hybridization). In the case of proteins, the probes usually are antibodies that react specifically with antigenic epitopes displayed by the target protein attached to the solid support. "

"In western blotting, the samples to be assayed are solubilized with detergents and reducing agents, separated by SDS-polyacrylamide gel electrophoresis, and transferred to a solid support (usually a nitrocellulose or PVDF filter), which may then be stained (eg with Ponceau S). The filter is subsequently exposed to unlabeled antibodies specific for the target protein. Finally the bound antibody is detected by one of several secondary immunological reagents (anti-immunoglobulin coupled to horseradish peroxidase or alkaline phosphatase), followed by enhanced chemiluminescence or enzymatic production of a colored precipitate. As little as 1-5 ng of an average sized protein can be detected by western blotting. "

Question 23: what is the difference between the alpha-amylase protein detected using an enzyme assay and the protein detected using a western blot?

Question 24: Estimate the size of the barley alpha-amylase? (hint, it is likely to be similar to the alpha-amylase found in other plants)

Material to be prepared by TA:

*SDS sample buffer (4x):

- 125 mM Tris HCl pH 6.8 (stock 1M)

- 10 % SDS (w/v) (stock 100%)
- 0.6 % (w/v) Bromphenolblue (stock 100%)
- 50% (v/v) Glycerol (stock 100%)
- add 10% (v/v) Beta-mercaptoethanol **before using** (stock 100%)

Give 250 µl to each group.

Remember to warm it up to ~50C for 20 min to dissolve SDS before use.

*10% Precise Protein Gels 12 wells x 30 ul e.a. Thermo (Pierce protein research product) Product# 25221. They are in the class fridge.

*Alpha-amylase standard, 12.4 mg/10 ml, give 100 µl to each group.

* Prestained protein standards low range (BioRad). (In PLB316 box in -20C, it is ready to use)

Protein	Size (Da)
Phosphorylase b	103,000
BSA	77,000
Ovalbumin	50,000
Carbonic anhydrase	34,300
Soybean trypsin inhibitor	28,800
Lysozyme	20,700

*Running buffer (see Pierce gel specifications) for 1L 10x:

121 g Tris-base
238 g Hepes
10 g SDS

Give 1L of 1x to each group.

*Transfer buffer for 1 L 10x:

390 mM glycine (MW 75.07 g/mole, 29.3 g/L)
480 mM Tris base (MW 121. 1 g/mole, 58.1 g/L)

for 1x, add 20% methanol (v/v) just before use.

Give 1L of 1x to each group.

*Coomassie Blue stain:

1 L: 500 ml Methanol
100 ml glacial acetic acid
400 ml H₂O
2g Coomassie Blue R-250 (not G-250)

Filter through a #1 whatman. Change filter several times. Make more than enough since it takes a long time to filter. Prepare 100 ml per group.

*Destaining solution: Make 3x as much as staining

1L : 1 L: 500 ml Methanol
100 ml glacial acetic acid
400 ml H₂O

*TBS-Tween for 1 L:

0.1 M 1M Tris-HCl pH 7.5 (stock 1M) = 100 ml 1M Tris-HCl pH 7.5
155 mM NaCl (stock 2.5M) = 62 ml NaCl (2.5M)
add H₂O to 1L

add 0.3% tween before use, mix well

Give 500 ml per group.

* 1 L pH 9.5 buffer: 100 mM Tris base (12.1 g/l)
100 mM NaCl (5.8 g/l)
5 mM MgCl₂ (1 g/l)

Adjust to pH 9.5.

Add 0.3% Tween-20 on day of use.

Give 100 ml to each group.

- * Give 2 empty tip boxes to each group (take from Farre lab), bring some extra boxes.
- * 1 Scissors per group.
- * Give each group 1 protein gel system and 1 power supply
- * Set up transfer apparatus with large power supply (does not work with the small ones), all gels will be transferred together.
- * Use a ½ plastic pipette (10 or 25 ml) for rolling out air bubbles in western set up.
- * Saranwapp (ask Jan or get from Biochemstore).
- * Shaker for membrane treatment with antibodies (we can use the shaking incubators by removing the flask holders).
- * Anti-alpha amylase antibody (TA will add 5-8 ml of antibody solution to each blot; **TA remember to collect the antibody after use!**)
- * Anti-rabbit alkaline phosphatase antibody: Dilute 1:1000 in TBS-Tween, give each group 10 ml.
- * Enzyme substrate for alkaline phosphatase secondary antibody, use Sigma tablets; B-5655, dilute 1 Tablet in 10 ml ddH₂O, this should be good for 2 blots.
- * **Remember to warm up heating block to 90C before class.**

Assay:

1. Transfer 120 µg of each protein extract to a 1.5 ml microfuge tube and adjust volume to 45 µl using distilled water. Prepare alpha-amylase standard: 12.5 mg/10 ml

Question 24: How many µl of each of your protein extracts do you need for 120 µg of total protein?

2. Add 15 µl SDS-PAGE 4x blue sample buffer, mix thoroughly, heat at 90C for 2 min in heating block. Your total volume will be 60 µl at a concentration 2 µg/ul. For the alpha-amylase standard add 15 µl sample buffer to 45 µl extract (12.5 mg/ml; final concentration 10 mg/ml).

Question 25: What does SDS do to your proteins?

Question 26: What does beta-mercaptoethanol do to your proteins?

Question 27: Why do you need to add both SDS and beta-mercaptoethanol to your extracts?

3. Centrifuge for 2 min at top speed.
4. Set up your gel running apparatus.
5. You will prepare 2 gels, here is the order of the samples

Gel1 (for coomassie staining)

- 5 µl of pre-stained standards
- 10 µl alpha-amylase standard
- 25 µl of each extract (50 µg total protein):
 - 0 days imbibition
 - 2 days imbibition
 - 3 days imbibition
 - 5 days imbibition

Gel2 (for western blot)

- 5 µl of pre-stained standards
- 2 µl alpha-amylase standard
- 3 µl of each extract (6 µg protein):
 - Buffer control + ethanol
 - Buffer + 50 nM GA₃
 - Buffer + 100 µM GA₃
 - Buffer +100 µM GA₃+ 2 µg/ml cycloheximide

Run at 100-110V, or ~ 40 mAmp.

5. For Coomassie staining:

- a. Transfer Gel 1 to the Coomassie staining solution for 30 min. Use an empty and clean tip box.
- b. Destain by washing gel twice for 30 min at 50C.

6. Western blot

- a. Transfer gel to the transfer buffer. Use an empty tip box.
- b. Prepare the membranes:

Note: be careful when handling the nitrocellulose membrane, use gloves and don't use pointy forceps. Don't remove the protecting sheets for the nitrocellulose membrane while cutting.

Cut 4 pieces of filter paper and one piece of nitrocellulose membrane (9x6 cm). Cut the filter papers slightly larger than your nitrocellulose membrane.

Soak them in transfer buffer (you can use another empty tip box for this): be careful when wetting the nitrocellulose membrane, do it **slowly** !

c. Set up your transfer in the following order:

Lower electrode (+)
2x filter paper
1x nitrocellulose membrane
gel
2x filter paper

Upper electrode (-)

*Add some extra transfer buffer to your setting, and remove air bubbles by rolling a 10 ml pipette slowly on your filter paper.

d. Transfer for 1 h at 0.8 mA per cm²

e. Turn off the power and remove your membrane from the western set up. Discard the filter papers and the gel in the regular trash. Remember to use gloves and do not use sharp objects to handle the membrane.

f. If we have time we will stain the membranes using Ponceau-S staining.

g. Place the membrane in a tip box with some Tris-buffered saline (TBS)-Tween buffer. Keep the membrane at 4C until the next class.

Development of the Western blot

All incubations are at room temperature with gentle shaking.

1. After transfer, mark the positions of the pre-stained standard proteins on your nitrocellulose (they may be partially obscured during the western development). Then put nitrocellulose in small pipette box lid and wash twice in Tris-buffered saline (TBS)-Tween, and for each wash use 20 ml for 5 min.
2. Add 5-8 ml primary antibody at the appropriate dilution (Usually 1:250). Incubate at least 45 min. **Don't discard the primary antibody.** Give it to the TA to be stored; the primary antibody can be used several times.
3. Wash twice with 20 ml TBS-Tween, each wash for 5 min.
4. Add 10 ml secondary antibody (anti-rabbit IgG coupled to alkaline phosphatase) at the correct dilution (usually 1:1000, dilute in TBS-tween), and incubate for at least 45 min.
5. Wash again once with TBS-Tween, then twice with pH 9.5 buffer (10-20 ml per wash, 2-3 min. each wash).
6. Add ~10 ml enzyme-substrate mix. Watch the color develop, and stop development when the colored bands appear. *Over-development will increase the background and will obscure differences between bands.*
7. Stop the reaction by rinsing with distilled water. (The color tends to darken for a min. or so after rinsing).
8. Photograph the Western blot with ruler alongside. (Alternatively, you can sandwich the nitrocellulose between saran wrap and photocopy).

9. Plot \log (MW) of prestained standards vs. distance migrated and calculate the MW of the proteins that are recognized by the antibody.

Results: Look carefully at the intensity of the bands and make as many conclusions as possible from these data. Compare to the SDS-PAGE gel stained with Coomassie.